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Lack of Z-DNA conformation in mitomycin-modified polynucleotides having inverted circular dichroism

(31P NMR/radioimmunoassay/antitumor agent/poly(dG-dC)·poly(dG-dC)/*Micrococcus lysodeikticus* DNA)

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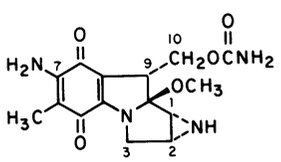
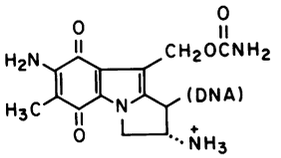
ABSTRACT **Poly(dG-dC)·poly(dG-dC) and *Micrococcus lysodeikticus* DNA were modified by exposure to reductively activated mitomycin C, an antitumor antibiotic. The resulting covalent drug-polynucleotide complexes displayed varying degrees of CD inversions, which are strikingly similar to the inverted spectrum observed with Z-DNA. The following criteria have been used to establish, however, that the inverted CD pattern seen in mitomycin C-polynucleotide complexes does not reflect a Z-DNA conformation. (*i*) The ethanol-induced transition of poly(dG-dC)·poly(dG-dC) from B to Z conformation is not facilitated but rather is inhibited by mitomycin C modification. This may be due to the presence of crosslinks. (*ii*) Radioimmunoassay indicated no competition for Z-DNA-specific antibody by any of the mitomycin C-modified polynucleotides. (*iii*) ^{31}P NMR of the complexes yielded a single relatively narrow resonance, which is inconsistent with the dinucleotide repeat characteristic of Z-DNA. Alternative explanations for the inverted CD pattern include a drug-induced left-handed but non-Z conformational change or the superposition of an induced CD onto the CD of B-DNA due to drug–base electronic interactions. These results illustrate the need for caution in interpreting CD changes alone as an indication of Z-DNA conformation.**

Mitomycin C (MC; structure I), an antibiotic and antitumor agent, binds covalently to DNA when activated by reductive metabolism *in vivo* or *in vitro* (1). Crosslinks between the complementary strands are also formed in this interaction as relatively rare events compared to the monofunctional binding (1).



Mitomycin C
(MC; I)



II

Some years ago, we noted a remarkable property of MC-DNA complexes: Their CD spectra showed a characteristic progressive change with increasing levels of MC binding (2). The same type of change, except much greater in magnitude, was seen with MC-poly(dG-dC)·poly(dG-dC) but not with MC-poly(dG)·poly(dC) or MC-RNA complexes, all compared at the same level of substitution by the drug (2). We noted that the CD changes and their specificity are strikingly similar to those occurring when poly(dG-dC)·poly(dG-dC) itself is submitted to high salt concentration (4 M NaCl or 1 M $MgCl_2$) as described by Pohl and Jovin some years earlier (3) (Fig. 1). These latter conditions induced a cooperative transition of the regular B form of poly(dG-dC)·poly(dG-dC) to a new conformation, unique to the alternating dG-dC sequence, the nature of which was unknown at the time except for its characteristic "inverted" CD (Fig. 1) and slightly altered UV spectrum. To account for the striking similarities between the MC-induced and salt-induced CD changes, we suggested (2), as one of several alternative explanations, that covalent binding of the positively charged MC ligand (structure II) to guanine residues of alternating dC-dG sequences induces and stabilizes the same new conformation as that induced by high salt concentration, whereas binding to guanine in other sequence environments does not produce such a change.

In the light of the discovery of crystalline Z-DNA, a left-handed double helix of unique structure (4), it soon became firmly evident that the salt-induced form of poly(dG-dC)·poly(dG-dC) in solution is also Z-DNA (5–7). Consequently, based on the above similarities, MC emerged as a strong candidate for a drug that is capable of inducing Z-DNA conformation upon binding to appropriate, alternating dG-dC sequences (4, 8).

In view of the extraordinary current interest in both structural aspects and biological significance of Z-DNA (see, e.g., ref. 9), it seemed timely to seek further evidence beyond the CD that would establish firmly whether this was indeed the case. MC is particularly interesting because the CD effect is seen not only with poly(dG-dC)·poly(dG-dC) but also with naturally occurring DNAs (2). As our objective, we probed both types of systems, specifically, MC-poly(dG-dC)·poly(dG-dC) and MC-*Micrococcus lysodeikticus* DNA complexes for Z-DNA properties by three different powerful methods: (*i*) comparison of the ethanol-induced B → Z transition (10) of the complex with that of poly(dG-dC)·poly(dG-dC), (*ii*) ^{31}P NMR, and (*iii*) radioimmunoassay (RIA).

MATERIALS AND METHODS

Materials used and their sources are as follows: mitomycin C, Bristol Laboratories (Syracuse, NY); oligo$(dA)_{10}$ and oligo$(dA)_{12-18}$, Collaborative Research (Waltham, MA); bacterial alkaline phosphatase and snake venom diesterase, Worthington–Millipore; and DNase I (pancreatic; type I), Sigma.

M. lysodeikticus DNA (Miles) was sonicated by using a Branson W140 sonicator and following a published protocol (11) and then was deproteinized by the Marmur procedure.

Poly(dG-dC)·poly(dG-dC) (P-L Biochemicals) was used without further purification. For the ^{31}P NMR experiments, it was

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Abbreviations: MC, mitomycin C; CD, circular dichroism; RIA, radioimmunoassay.

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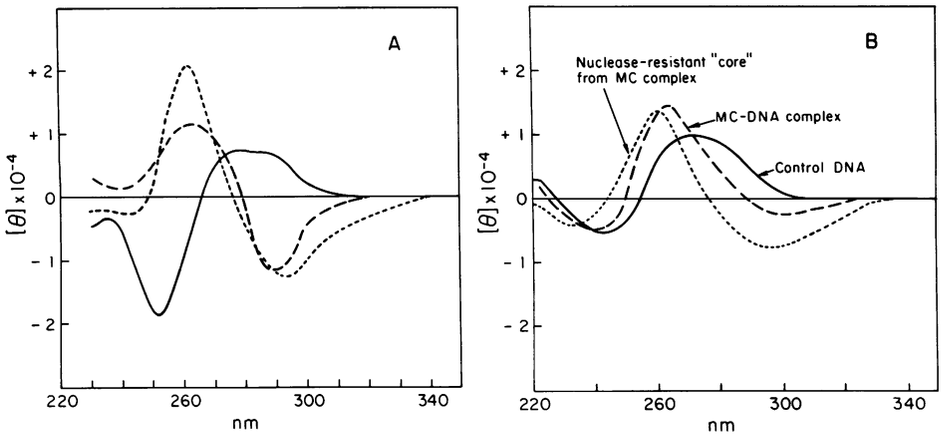

FIG. 1. CD spectra. (*A*) ——, poly(dG-dC)·poly(dG-dC) in 0.017 M sodium phosphate, pH 7.5/0.1 mM EDTA; – – –, same polymer in the same buffer with 4 M NaCl; ---, MC-poly(dG-dC)·poly(dG-dC) complex (binding ratio, 0.25) in 0.017 M sodium phosphate, pH 7.5/0.1 mM EDTA (replotted from figure 8 in ref. 2). (*B*) – – –, *M. lysodeikticus* DNA-MC complex (binding ratio, 0.15); ---, nuclease-resistant MC-oligonucleotide core from the same complex (binding ratio, 0.25); ——, control DNA. All were in 0.012 M Tris, pH 7.4/1 mM EDTA.

sonicated exactly as described (12). Unsonicated polymer was used in all other experiments.

MC-poly(dG-dC)·poly(dG-dC) complexes with various binding ratios were prepared as published (2). MC–*M. lysodeikticus* DNA complexes were made by the same general procedure.

Binding ratios, defined as moles of antibiotic bound per mole of mononucleotide unit, were determined by the UV absorbance method (13).

Nuclease digestion of MC–*M. lysodeikticus* DNA complexes (binding ratio, 0.15) was carried out by incubating 850 A_{260} units of the complex in 170 ml of 0.14 M Tris/0.04 M $MgCl_2$, pH 7.0, with a mixture of pancreatic DNase (10 units per A_{260} unit of DNA), snake venom diesterase (0.14 units per A_{260} unit of DNA), and alkaline phosphatase (0.14 units per A_{260} unit of DNA) for 18 hr at 30°C. EDTA was added to 0.025 M, and the mixture was deproteinized by four extractions with $CHCl_3$/isoamyl alcohol, 24:1 (vol/vol), followed by chromatography over a Sephadex G-25 column (5 × 55 cm) in 0.02 M NH_4HCO_3 buffer. The void volume fraction contained the nuclease-resistant "core" material. This was further purified from protein by chromatography on DEAE-cellulose column (DE-52, Whatman; 1.5 × 17 cm). The protein was eluted first with 0.2 M NaCl/0.06 M Tris, pH 7.4, and then the core was eluted with 0.6 M NaCl/0.06 M Tris, pH 7.4. Its size range was estimated by comparison of its elution volume with those of oligo$(dA)_{12-18}$ and oligo$(dA)_{10}$ on a Sephadex G-100 column. Base analysis was carried out by hydrolysis in 70% $HClO_4$ at 100°C, followed by neutralization, centrifugation, and separation of the bases in the supernatant by HPLC (Ultrasphere-octadecylsilica reverse-phase-type column, Beckman; solvent, 0.03 M potassium phosphate, pH 6.3). Base ratios were calculated from comparison of the peak areas.

CD spectra were recorded with a Cary 60 spectrophotometer, as described (2). Molar ellipticity ($[\theta]$) was calculated per nucleotide residue.

The extent of CD inversion of MC-poly(dG-dC)·poly(dG-dC) complexes is calculated from $\Delta[\theta]$ ($[\theta]_{complex}$ minus $[\theta]_{control}$) at 260 and 290 nm. $\Delta[\theta]$ of the saturation complex (binding ratio, 0.22–0.25) is regarded as the value for 100% inverted CD. (The 0.22–0.25 ratio represents substitution of every second base pair by MC, and it is not possible to introduce more covalently bound MC.) The CD of this complex is given in Fig. 1*A* along with the Z form of poly(dG-dC)·poly(dG-dC). Calculated extents of inversion (% inversion) at 260 and 290 nm agree closely.

^{31}P NMR spectra were obtained with a Nicolet 360 wide-bore spectrometer tuned to a frequency of 121.47 MHz. Spectra at 8 K were collected with a pulse width of 60° and a pulse repetition rate of 1.5 sec. Two-level broad-band irradiation with 7- and 3-W power was applied to the sample for full proton decoupling.

Competitive RIA with ^3H-labeled Z-DNA was carried out as described (14) under conditions of moderate and high ionic strengths (60 mM sodium phosphate/30 mM EDTA, pH 8.0, with 0.2 M or 4 M NaCl). Brominated poly(dG-dC)·poly(dG-dC) was prepared as described (14) and showed the CD of Z-DNA under both ionic conditions. The polymer was labeled by nick-translation with ^{3}[H]dGTP and ^{3}[H]dCTP as described (14).

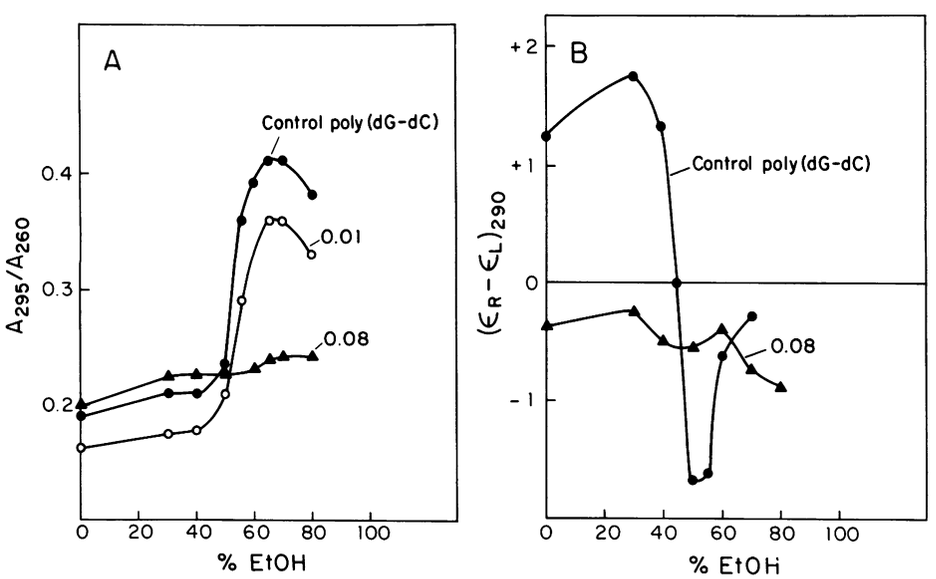

FIG. 2. Ethanol-induced B → Z transition of poly(dG-dC)·poly(dG-dC) (●) and its MC complexes (○, ▲) as monitored by changes in the UV spectra (*A*) and by CD change at 290 nm (*B*), both with increasing percentage (wt/wt) of ethanol in 1 mM sodium phosphate, pH 7.2/0.1 mM EDTA. The numbers near the curves indicate binding ratios.

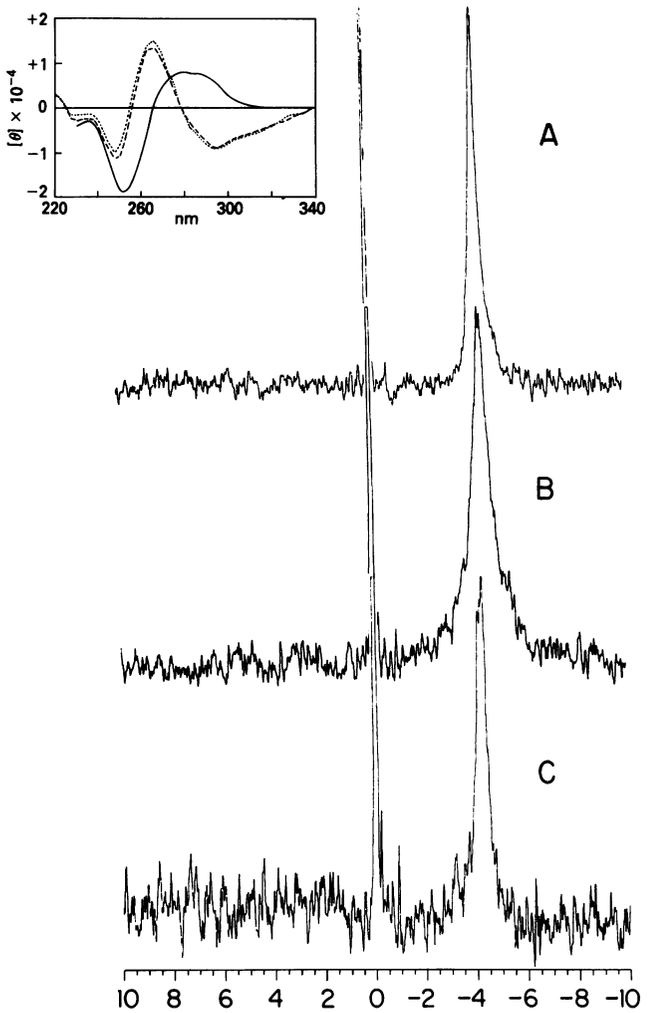

FIG. 3. ^{31}P NMR at 121.47 MHz of sonicated control poly(dG-dC)·poly(dG-dC) (*A*), MC-sonicated poly(dG-dC)·poly(dG-dC) complex (binding ratio, 0.17) (*B*), and MC-oligonucleotide core from *M. lysodeikticus* DNA (binding ratio, 0.23) (*C*). All spectra were taken in 0.012 M Tris, pH 7.4/1 mM EDTA (20% ^2H_2O) at 3.3–4.0 mM nucleotide. For these spectra, the number of scans accumulated are 10,240 (*A*), 15,360 (*B*), and 5,120 (*C*). Exponential line broadening of 5 Hz was applied to all spectra. Chemical shifts are reported relative to the internal reference, trimethyl phosphate. (*Inset*) CD spectra of samples *A* and *B* in the same buffer. ——, Sample *A* at 0.1 mM nucleotide; – – –, sample *B* at the same concentration; and ---, sample *B* at 1.2 mM nucleotide. (CD was measured at 1-mm path length.)

Binding of ^3H-labeled Z-DNA in 0.2 M NaCl with or without various amounts of competitor was tested with a 1:1,500 dilution of a serum that reacted with Z-DNA but not with B-DNA even at high serum concentration (14). For experiments in 4 M NaCl, a 1:2,400 dilution of the serum was used.

RESULTS

Three distinct types of MC-polynucleotide complexes were selected for study:

(*i*) MC-poly(dG-dC)·poly(dG-dC) with various binding ratios. The CD of these complexes is progressively inverted with increasing binding ratio as shown previously (2). Fig. 1*A* shows the CD spectra at the limiting binding ratio (0.22–0.25).

(*ii*) MC-*M. lysodeikticus* DNA with various binding ratios. The CD of the complex (binding ratio, 0.15) is shown in Fig. 1*B* together with that of control DNA, indicating some inversion.

(*iii*) Nuclease-resistant MC-oligonucleotide core from MC-*M. lysodeikticus* DNA complexes. This was obtained, upon digestion of the complex (binding ratio, 0.10–0.15) with a mixture of nucleases, in 6–12% yield with the following properties: binding ratio, 0.22–0.26; size range, 10–20 nucleotides; CD, highly inverted (Fig. 1*B*); and base composition, 42.5% G, 43.1% C, 7.5% T, and 6.9% A. The latter data clearly indicate that the core had the composition of a double-stranded DNA with 86% G+C content. In summary, it was a mixture of oligonucleotides enriched in G+C (86%) relative to the parent DNA (72%) and enriched in bound MC, containing the saturation amount of drug (binding ratio, 0.22–0.26). Its CD had a greatly increased negative minimum at 293 nm and a further shift of the positive maximum to lower wavelength, compared to the undigested complex (Fig. 1*B*), and 80% inversion was calculated when compared to the complex in Fig. 1*A*. In view of these properties, it appeared possible that the core is a mixture of short pieces of Z-DNA.

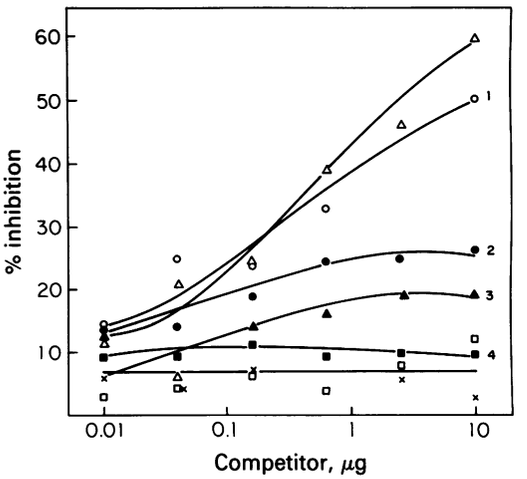

FIG. 4. Competitive RIA of various MC-polynucleotide complexes with ^3H-labeled brominated poly(dG-dC)·poly(dG-dC) in 4 M NaCl/0.06 M sodium phosphate, pH 8.0/0.03 M EDTA. Inhibitors: △, poly(dG-dC)·poly(dG-dC); ○, MC-poly(dG-dC)·poly(dG-dC) complex (binding ratio, 0.04; curve 1); ●, MC-poly(dG-dC)·poly(dG-dC) complex (binding ratio, 0.10; curve 2); ▲, MC-poly(dG-dC)·poly(dG-dC) (binding ratio, 0.19; curve 3); ■, MC-*M. lysodeikticus* DNA complex (binding ratio, 0.07; curve 4); □, MC-oligonucleotide core from *M. lysodeikticus* DNA (binding ratio, 0.23); and x, *M. lysodeikticus* DNA.

Ethanol-Induced B → Z Transition of Unmodified and MC-Modified poly(dG-dC)·poly(dG-dC). The transition was monitored by two methods, first used by Pohl (10): change of A_{295}/A_{260} (Fig. 2*A*) and change of $(\varepsilon_R - \varepsilon_L)$ at 290 nm with increasing ethanol concentration (Fig. 2*B*). The complex with a binding ratio of 0.01 required the same or slightly higher ethanol concentration for transition midpoint than did unmodified poly(dG-dC)·poly(dG-dC) (54% vs. 52%) (Fig. 2*A*), whereas the complex with a binding ratio of 0.08 showed essentially no transition even though its CD (not shown) was only about 40% inverted relative to that of the saturation complex shown in Fig. 1*A*. The results in Fig. 2*B* (the descending part of the control curve is the B → Z transition) confirm the lack of B → Z transition of this complex. In summary, the transition is not facilitated but rather is inhibited with increasing binding ratio of the MC-poly(dG-dC)·poly(dG-dC) complex.

^{31}P NMR. *MC-sonicated poly(dG-dC)·poly(dG-dC) complex (binding ratio, 0.17).* Figs. 3 *A* and *B* show the spectra of the sonicated poly(dG-dC)·poly(dG-dC) control and MC bound to poly(dG-dC)·poly(dG-dC). Like the control, the spectrum of the complex shows a single peak centered at 4.3 ppm upfield from the internal trimethylphosphate standard. The CD for this complex (Fig. 3 *Inset*) shows 68% inversion relative to that of the saturated complex (Fig. 1*A*). Based on a binding ratio of 0.17 MC per nucleotide, we could expect a second peak at 2.8

ppm, characteristic of Z-DNA (5, 15, 16), with a peak intensity of 34% of the main band. Although this peak would easily be detected above background, no such peak is apparent. Therefore, the spectra do not support a conversion to Z-DNA. Some broadening and a slight peak splitting (≈10 Hz) are evident, however, with MC binding; the linewidths for sonicated poly(dG-dC)·poly(dG-dC) and MC-poly(dG-dC)·poly(dG-dC) complex are 49 Hz and 97 Hz, respectively.

MC–oligonucleotide core from M. lysodeikticus DNA (binding ratio, 0.26). The ^{31}P NMR spectrum of this core complex (Fig. 3*C*) also showed a single resonance at 4.2 ppm with a linewidth of 61 Hz and some fine splitting. Again, although the CD of the substance (Fig. 1*B*) showed an inverted spectrum characteristic of Z-DNA, there was no indication of a second peak at 2.8 ppm in the ^{31}P NMR spectrum.

Lack of concentration dependence of the CD. Because the NMR experiments are conducted at much higher polynucleotide concentration than that used for CD experiments (3.3 mM vs. 0.1 mM in our case), we were concerned about the possibility that the CD of the NMR samples might be different from the CD of the dilute samples due to a concentration-dependent conformational change (cf. ref. 17). No appreciable difference was seen, however, between the CDs of the usual 0.1 mM and a 1.2 mM sample of the MC-poly(dG-dC)·poly(dG-dC) complex, even though the latter corresponds to a 12-fold increase in polynucleotide concentration (Fig. 3 *Inset*). The latter concentration is of the same order of magnitude as that of the NMR samples (3.3 mM). Furthermore, integration of the ^{31}P NMR spectrum of MC-poly(dG-dC)·poly(dG-dC) yielded values consistent with the expected polynucleotide concentration, suggesting that there was no aggregation or precipitation at high concentration. These results indicate that the CD and NMR spectra reflect the same conformation.

Competitive RIAs with ^{3}H-Labeled Z-DNA. In 0.2 M NaCl/0.06 M sodium phosphate, pH 8.0/0.03 M EDTA, none of the following samples showed any competition for Z-DNA-specific antibody with ^{3}H-labeled brominated poly(dG-dC)·poly(dG-dC) (14): control poly(dG-dC)·poly(dG-dC), three different MC-poly(dG-dC)·poly(dG-dC) complexes (binding ratio, 0.04, 0.10, and 0.19), control *M. lysodeikticus* DNA, MC-*M. lysodeikticus* DNA complex (binding ratio, 0.07), and MC-oligonucleotide core from *M. lysodeikticus* DNA (binding ratio, 0.23).

In 4 M NaCl/0.06 M sodium phosphate, pH 8.0/0.03 M EDTA, competition was observed by control poly(dG-dC)·poly(dG-dC) and each of its three complexes above (Fig. 4). However, the more the polynucleotide was modified by MC, the less it was able to compete with the Z-DNA of the assay; the *M. lysodeikticus* DNA samples were all negative (Fig. 4).

RIAs with a Monoclonal Antibody H241. The monoclonal antibody H241 (unpublished data) reacts with native or denatured DNA and with poly(dG-dC)·poly(dG-dC). In the latter case, the antibody prefers the B form and binds more strongly to it than to Z-DNA or calf thymus DNA. MC-poly(dG-dC)·poly(dG-dC) complexes and unsubstituted poly(dG-dC)·poly(dG-dC) were tested as competitors of native calf thymus [^{3}H]DNA for this antibody in 0.2 M NaCl/0.06 M sodium phosphate, pH 8.0/0.03 M EDTA. The complexes with binding ratios of 0.04 and 0.10 displayed competitions identical to that by unsubstituted poly(dG-dC)·poly(dG-dC). The complex with the highest binding ratio (0.19) still inhibited completely but required 5 times higher concentration.

DISCUSSION

The ethanol-induced cooperative B → Z transition of poly(dG-dC)·poly(dG-dC) (10) has provided a useful method for probing various covalent modifications of poly(dG-dC)·poly(dG-dC) for their ability to stabilize the Z-DNA structure. The method consists simply of comparison of the ethanol concentration required for the transition of modified and unmodified poly(dG-dC)·poly(dG-dC). A lower concentration in the former case indicates that the modification promotes the Z structure. Thus, poly(dG-dC)·poly(dG-dC) modified by the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene at 3% of the bases required 45% ethanol, compared to 50–55% ethanol for the control (18), whereas 6.6% modification of the bases lowered the requirement to 25% ethanol concentration for the transition (19). Similar trends were observed in the case of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (20), poly(m^{7}dG-dC)·poly(m^{7}dG-dC) (21), and poly(dG-dC)·poly(dG-dC) modified by a platinum compound (17). In each of these examples, the increased stability of Z-DNA was confirmed by other criteria as well. However, the mitomycin-induced modification of poly(dG-dC)·poly(dG-dC) does not facilitate the transition (Fig. 2 *A* and *B*). Indeed, the results show that it inhibits it. This indicates that some blocks exist to the spread of the cooperative B → Z change occurring at high ethanol concentration.

The RIAs with ^{3}H-labeled Z-DNA indicated very clearly that under physiological conditions the Z-DNA-specific antibody did not recognize even the most highly substituted MC-poly(dG-dC)·poly(dG-dC) complex (binding ratio, 0.19), although the CD of this complex is essentially fully inverted under similar conditions (cf. Fig. 1*A*). This result in itself would not be sufficient to rule out Z-DNA because it might be argued that the "bulky substituent" MC interferes with antibody recognition. However, evidence against this argument comes from testing the complexes with a monoclonal antibody, H241, which reacts with native or denatured DNA and with poly(dG-dC)·poly(dG-dC). In the latter case, the antibody prefers the B form and binds more strongly to it than to Z-DNA or calf thymus DNA (unpublished data). The MC-poly(dG-dC)·poly(dG-dC) complexes with binding ratio 0.04 and 0.10 each showed identical reactivity toward this antibody with that of unsubstituted poly(dG-dC)·poly(dG-dC).

One can conclude from these results, that the bound MC, at least up to a substitution level of 0.10 (20% of the guanines substituted!) did not hinder access to the backbone of DNA, nor did the complexes show any Z character. At the highest substitution, it is possible that there was some hindrance but not enough to account for the failure to inhibit the anti-Z-DNA. It is also possible that the highest substitution causes a conformational change that is not Z but still makes it a less effective inhibitor of H241 than samples 1 or 2.

Further, a strong indication that the results of the RIAs are not a function of substituent hindrance is provided by the ethanol transition studies. They show independently that increasing binding ratio of MC results in progressive inhibition of the B → Z transition (Figs. 2 and 3). This confirms that the results of the immunoassays in 4 M NaCl (Fig. 4) indicate the same phenomenon—i.e., that increasing binding ratio of the MC-poly(dG-dC)·poly(dG-dC) complex progressively inhibits the transition to the Z structure under the high salt conditions. This interpretation provides a striking consistency between the results of the two different types of experimental approaches (physicochemical and immunological.)

From this it may be concluded that a structural barrier is present in MC-modified poly(dG-dC)·poly(dG-dC), preventing the formation of Z-DNA under the usual conditions. [Recently a similar inhibition of the B→Z transition was observed in aflatoxin B_1–poly(dG-dC)·poly(dG-dC) complexes (22).] This is noticeable even at the low levels of 1, 4, and 8% base substitutions. This may be a general effect of crosslinking agents and could have biological significance.

The *M. lysodeikticus* DNA samples modified by MC also

showed no Z structure when tested with the Z-DNA-specific antibody. This was true for the nuclease-resistant MC-oligonucleotide core from the DNA as well, even though this substance, which has 80% inverted CD and 86% G+C content, appeared to be a most promising candidate for Z-DNA originally.

^{31}P NMR experiments provide a unique probe for the dinucleotide repeat, characteristic of Z-DNA (5, 15, 16). The MC-sonicated poly(dG-dC)·poly(dG-dC) sample had a calculated 86% inversion of CD; if this were due to Z-DNA, one would expect two phosphorus resonances at ≈2.8 and ≈4.3 ppm upfield from the internal standard trimethylphosphate in an intensity ratio of 34:66. The appearance of the former resonance would indicate the inequivalence of neighboring phosphates in Z-DNA (5, 15, 16). Only one peak is evident, however, at the position of that of the control (4.30 ppm). Similarly, the core (calculated CD inversion, 80%), if Z-DNA, may be expected to display the two peaks in an approximate intensity ratio of 40:60; but again, only the usual DNA peak at 4.20 ppm is present. These results support the lack of Z-DNA upon modification by MC. Because the ^{31}P NMR shifts for Z-DNA have been reported mostly for unmodified poly(dG-dC)·poly(dG-dC), extension to covalently modified polynucleotides should be exercised with some caution until more cases of such correlations become known. Thus far a positive correlation has been seen in two cases (see refs. 17 and 23).

What is then the origin of the inversion of the CD in these drug-polynucleotide complexes? There are two *a priori* possibilities. (*i*) It is due to true conformational changes of the polynucleotide, but no Z-DNA is involved. (*ii*) The CD change is the result of electronic interaction between base chromophores and covalently bound mitomycin, giving rise to additional Cotton effects (induced CD) (24), which are superimposed on the usual CD of B-DNA. Both alternatives have been discussed in our earlier paper (2). The first one gained new interest in view of the recent discovery of several types of left-handed DNA structures distinct from Z-DNA in solution, all characterized by inverted type CD spectra and alternating dG-dC sequences (25). A test for the change in handedness, namely relaxation of appropriate supercoiled topoisomers (25) cannot be applied unambiguously to MC-modified plasmids because relaxation of the latter has been reported to take place as a result of some single-strand cleavage due to superoxide formation during the usual procedure of reductive activation of MC (26). That conformational changes do exist in DNA modified by MC was reported recently from one of our laboratories (27). Hydrodynamic and electron microscopic evidence indicated greatly increased flexibility of limited regions in MC-calf thymus DNA complexes. Further indications for a deviation from the B-DNA conformation was provided by transient electric dichroism studies (28). Although the hypothesis of Z-DNA is no longer feasible to explain these effects, left-handed DNA remains a distinct possibility. A test that discriminates between right-handed and left-handed DNA in solution is under development (29) and may be applicable to this problem.

Induced CD, a second potential cause of the CD inversion of MC-polynucleotide complexes, receives some supportive evidence from recent chemical results: a mitomycin-deoxyguanosine adduct was recently isolated that had an intense CD spectrum with negative and positive Cotton effects resembling somewhat the CD of Z-DNA (30). Thus, the inverted CD of MC-poly(dG-dC)·poly(dG-dC) could result from the sum of the CD of B-DNA and of the extrinsic CD of deoxyguanosine-MC adducts. It should be noted that MC does not intercalate between base pairs (27) and, therefore, the electronic interaction, if any, does not arise from intercalative stacking. Somewhat similar CDs of adducts between 7,12-dimethylbenzanthracene and guanosine (31) as well as between *N*-2-acetylaminofluorene and some mono- and dinucleoside-phosphates (32) have been reported in the literature.

The present finding that Z-DNA-like inverted CD can result from factors other than the presence of Z-DNA underscores the need for caution in interpreting such CD changes. The power of the particular combination of the three tests for Z-DNA used here is clearly demonstrated and it should be equally useful and conclusive for negative and positive cases.

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